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# Effects of early life stress on adult male aggression and hypothalamic vasopressin and serotonin

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**Keywords:** anterior hypothalamus, HPA axis, maternal separation, rat, resident-intruder

## Abstract

Early life stress in humans enhances the risk for psychopathologies, including excessive aggression and violence. In rodents, maternal separation is a potent early life stressor inducing long-lasting changes in emotional and neuroendocrine responsiveness to stress, associated with depression- and anxiety-like symptoms. However, effects of maternal separation on adult male aggression and underlying neurobiological mechanisms remain unknown. Therefore, we investigated the effects of maternal separation on adult intermale aggression in Wistar rats and on hypothalamic arginine vasopressin (AVP) mRNA expression, and AVP and serotonin (5-HT) immunoreactivity, as both AVP and 5-HT have been implicated in stress-coping and aggression. We showed that maternal separation induced depression-like behaviour (increased immobility) and higher adrenocorticotropin hormone responses to an acute stressor (forced swimming). Intermale aggression (lateral threat, offensive upright and keep down) was significantly higher in maternally separated rats compared with control rats. AVP mRNA expression and AVP immunoreactivity were higher in the hypothalamic paraventricular and supraoptic nuclei upon resident-intruder test exposure, whereas 5-HT immunoreactivity was decreased in the anterior hypothalamus of maternally separated rats. Moreover, 5-HT immunoreactivity in the anterior hypothalamus and supraoptic nucleus correlated negatively with aggression. These findings show that exposure to early life stress increases adult male aggression in an animal model of maternal separation. Furthermore, the maternal separation-induced changes in hypothalamic AVP and 5-HT systems may underlie these behavioural alterations.

## Introduction

Individuals with a history of childhood maltreatment (child abuse, physical and emotional neglect, parental loss) often show impulsive aggression, violent and/or criminal behaviour, and antisocial personality symptoms (Widom, 1989; Dodge *et al.*, 1990; Patterson, 1995; Loeber & Stouthamer-Loeber, 1998; Barnow, 2001; Barnow & Freyberger, 2003; Barnow *et al.*, 2004). Childhood maltreatment has also been associated with other forms of adult psychopathologies, like depression and anxiety disorders (Agid *et al.*, 1999; Heim & Nemeroff, 2001; Newport *et al.*, 2002). An animal model for early adverse experience is the separation of pups from their mother for 3 h daily during the first 2 weeks of life (maternal separation, MS) (Plotsky & Meaney, 1993; Newport *et al.*, 2002). MS has been shown to chronically impair emotional and neuroendocrine responses. For example, MS rats are characterized by increased anxiety-related behaviours (Wigger & Neumann, 1999; Huot *et al.*, 2002; Kalinichev *et al.*, 2002; Romeo *et al.*, 2003), increased corticotropin-releasing hormone (CRH) mRNA expression in the hypothalamic paraventricular nucleus (PVN) (Plotsky & Meaney, 1993), and elevated plasma adrenocorticotropin hormone (ACTH) concentrations in response to an acute stressor (Plotsky & Meaney, 1993; Ladd *et al.*, 1996; Wigger & Neumann, 1999; Liu *et al.*, 2000; Huot *et al.*, 2002; Kalinichev *et al.*, 2002). Interestingly, hypothalamic–pituitary–adrenocortical

(HPA) axis abnormalities in humans and rodents have often been associated with changes in male aggression (Lyons-Ruth, 1996; McBurnett *et al.*, 2000; de Kloet, 2003; Haller *et al.*, 2004).

Although it is generally accepted that early life trauma is a universal risk factor for excessive aggression in adult humans, animal models studying developmental stress-induced changes in aggression and their underlying neurobiological mechanisms are still lacking. The present study investigated the consequences of MS on adult male aggression in Wistar rats. Furthermore, human and animal studies have suggested a role for arginine vasopressin (AVP) and serotonin (5-HT) in various aspects of emotional behaviours, including anxiety (Baldwin & Rudge, 1995; Landgraf *et al.*, 1998; Lesch *et al.*, 2003; Bielsky *et al.*, 2004; Gordon & Hen, 2004; Griebel *et al.*, 2005; Veenema *et al.*, 2005) and aggression (Ferris, 1996; Coccaro *et al.*, 1998; Koolhaas *et al.*, 1998; Nelson & Chiavegatto, 2001; Olivier, 2005). In particular at the level of the hypothalamus, AVP was shown to increase anxiety (Keck *et al.*, 2003; Wigger *et al.*, 2004) and aggression (Ferris, 1992; Delville *et al.*, 1996a). Hypothalamic 5-HT seems to diminish aggression, likely via inhibiting local AVP actions (Delville *et al.*, 1996a; Ferris, 1996; Ferris *et al.*, 1997). Therefore, MS-induced changes in hypothalamic AVP and 5-HT systems were also investigated. In the first experiment the application of the MS model in our laboratory was validated by measuring in adult male rats MS-induced changes in depression-like behaviour and neuroendocrine (plasma ACTH, corticosterone and testosterone) responses to an acute stressor (forced swimming). In the second experiment, MS-induced alterations in AVP mRNA expression were investigated. In the third experiment,

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adult male rats were tested for MS-induced alterations in home-cage aggression using the resident-intruder (RI) test and in AVP and 5-HT immunoreactivity.

## Materials and methods

### Animals

After 1 week of habituation in our laboratory facility, female and male Wistar rats (Charles River, Sulzfeld, Germany) were mated for 5 days. During the last week of gestation, female rats were individually housed in standard rat cages (42 × 27 × 18 cm), and maintained under standard laboratory conditions (12 : 12 light : dark cycle, lights on at 06.00 h, 22 °C, 60% humidity, food and water *ad libitum*). The animal studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the Government of Bavaria and the guidelines of the NIH.

### MS procedure

On the day after parturition, i.e. on postnatal day 1, each litter was culled to eight–10 pups (in each nest two–four females). Pups were separated daily between 09.00 h and 12.00 h (noon) from the mother for 3 h from postnatal day 1 to 14. Dams were removed from the home-cage and placed into a separate individual cage until the end of the 3-h separation period. Pups were then removed as complete litters from the nest, transferred to an adjacent room and put into a small box filled with bedding, which was placed on a heating pad maintained at 30–33 °C. After the 3-h separation period, the pups were returned to the home-cage followed by reunion with the dam. Non-separated control litters were left undisturbed, except for change of bedding at postnatal day 1, 7 and 14. Pups were weaned at postnatal day 21 and housed in groups of four–five of the same gender and treatment until the start of the experiments.

### Experimental design

Three experiments were performed. In experiment 1, effects of MS on adult behavioural and neuroendocrine reactivity to an acute stressor (forced swimming) were investigated. Experiment 2 assessed the effects of MS on AVP mRNA expression in the PVN and supraoptic nucleus (SON) under basal conditions and after exposure to the RI test. Experiment 3 assessed the effects of MS on adult male aggression during the RI test, and on AVP and 5-HT immunoreactivity in several hypothalamic regions under basal conditions and after RI exposure. For experiments 1 and 3, male pups were taken from 13 litters (six control and seven MS). For experiment 2, male pups were taken from 14 litters (seven control and seven MS). No more than two males per litter were used for each experiment group.

### Experiment 1: MS effects on behavioural and neuroendocrine reactivity

#### Forced swim test

At the age of 11 weeks, active vs. passive stress-coping in MS ( $n = 10$ ) and control ( $n = 8$ ) rats was tested during the forced swim test carried out between 10.00 and 11.00 h. The procedure was a modified version of the test described before (Porsolt *et al.*, 1977). Rats were forced to swim inside a Plexiglas cylinder (diameter, 30 cm; height, 50 cm) filled with water (25 °C) for 7 min. As an indicator of passive stress-coping, the duration of immobility (floating in the water

without struggling, making only those movements necessary to keep its head above the water) and the latency time for immobility were recorded. As an indicator of active stress-coping, the duration of swimming (moving around in the cylinder, active swim movements) and climbing (movements with the forepaws in and out of the water, usually directed against the wall) were also recorded.

### Jugular vein surgery and blood sampling

One week after the forced swim test, the same MS and control rats were implanted with chronic jugular vein catheters under isoflurane anaesthesia and using sterile procedures as described before (Neumann *et al.*, 1998). Following surgery, rats were singly housed in experimental cages (40 × 24 × 35 cm) and were handled each day to reduce non-specific stress responses during the experiments. Five days after surgery, at 09.00 h, the catheter of each rat was attached to an extension tube connected to a 1-mL plastic syringe filled with sterilized heparinized 0.9% saline (30 IU/mL, Heparin-Natrium, Ratiopharm, Ulm, Germany). The rats were then left undisturbed for 2 h. Following two basal blood samplings (30 min and 0 min prior to stress exposure), rats were forced to swim for 5 min and were then returned to their home-cage, and additional blood samples were taken 5, 15, 30, 60 and 90 min after the onset of the swim stressor. The 0.2-mL blood samples were immediately replaced by sterile 0.9% saline. Blood samples were collected from MS ( $n = 8$ ) and control ( $n = 7$ ) rats.

### Radioimmunoassay for plasma ACTH, corticosterone and testosterone

All blood samples were collected in chilled EDTA-coated tubes (Sarstedt, Nümbrecht, Germany) containing 10 µL aprotinin (Trasyol, Bayer AG, Leverkusen, Germany) and centrifuged at 2600 *g* for 10 min at 4 °C. Plasma aliquots were stored at –20 °C until assayed. Plasma ACTH (50 µL), corticosterone (10 µL) and testosterone (50 µL) were determined using commercially available radioimmunoassays (ICN Biomedicals, Costa Mesa, CA, USA). Detection limits for ACTH, corticosterone and testosterone were 4.0 pg/mL, 10 ng/mL and 0.6 ng/mL, respectively.

### RI test

Two weeks before the start of experiments 2 and 3 (see below), the 12 : 12 h light : dark cycle was switched to lights off at 13.00 h. Rats underwent the RI tests at the age of 14–16 weeks and weighed 350–450 g. Each rat was housed in an experimental cage (40 × 24 × 35 cm) either single for 2 days (experiment 2) or together with a female Wistar rat for 2 weeks (experiment 3). RI tests were carried out during the beginning of the dark cycle (between 14.00 and 16.00 h). During the RI test, the resident MS or control male was exposed in its home-cage to a slightly smaller (20–50 g lighter) unfamiliar male Wistar rat for 10 min. In experiment 2, rats were exposed to a single RI test. In experiment 3, rats underwent three RI tests carried out on consecutive days, and the full behavioural profile was recorded. Thirty minutes before each RI test the female was removed from the resident's home-cage and was returned afterwards. The tests were videotaped and the behavioural scoring was done using Eventlog (version 1.0, October 1986, R. Hedersen) by a researcher blinded to the treatment condition. The following parameters related to male aggression were scored: attack latency time, number of attacks, lateral threat, clinch, offensive upright and keep down. The latter four behavioural parameters were summarized as total aggressive behaviour. Furthermore, social behaviour (consisting of investigating opponent, anogenital sniffing, mount), exploration and self-grooming were scored.

### Experiment 2: MS effects on AVP mRNA expression

At the age of 16 weeks, MS ( $n = 8$ ) and control ( $n = 8$ ) rats underwent a RI test, and 1 h later rats were decapitated under CO<sub>2</sub> anaesthesia, brains were rapidly removed, quickly frozen in ice-cold isopentane and stored at  $-80^{\circ}\text{C}$  for subsequent AVP *in situ* hybridization. The 1 h time point for measuring changes in AVP mRNA expression upon RI test exposure was chosen based on previous studies showing alterations in AVP expression patterns 1 h after exposure to several different stressors (Dent *et al.*, 2000; Givalois *et al.*, 2004; Itoi *et al.*, 2004; Kawasaki *et al.*, 2005; McDougall *et al.*, 2005). Another group of MS ( $n = 8$ ) and control ( $n = 8$ ) rats were decapitated under similar conditions, but without the preceding RI test in order to measure AVP mRNA expression under basal conditions.

#### AVP *in situ* hybridization

Brains were sectioned coronally at 16  $\mu\text{m}$  on a cryostat and thaw mounted onto poly-L-lysine-coated slides and stored at  $-80^{\circ}\text{C}$ . Brain sections were matched for level among the groups. The same number of brain slices for each animal was used.

The hybridization protocol was adopted from De Vries *et al.* (1994). Briefly, slides were fixed in 4% paraformaldehyde, acetylated in 0.25% triethanolamine/acetic anhydride and dehydrated through a series of graded ethanols. Hybridization was performed using a specific 48-mer, <sup>35</sup>S-labelled oligonucleotide probe: 5'-GCA-GAA-GGC-CCC-GGC-CGG-CCC-GTC-CAG-CTG-CGT-GGC-GTT-GCT-CCG-GTC-3' (Bosch *et al.*, 2006). Sections were incubated in hybridization solution [50% formamide, 10% dextran sulphate, 2  $\times$  standard sodium citrate (SSC), 2 mg/mL yeast tRNA, 10 mM dithiothreitol, 5  $\times$  Denhardt's]. The probe was applied to each section at a concentration of 10<sup>6</sup> cpm/slide in 200  $\mu\text{L}$  hybridization solution. The sections were hybridized overnight at 50  $^{\circ}\text{C}$  in a humidified chamber. Then, sections were washed three times in 1  $\times$  SSC at 50  $^{\circ}\text{C}$ , washed in 1  $\times$  SSC at room temperature, dehydrated in a graded series of ethanol and air dried. Hybridized sections were exposed to X-Omat film (Kodak, Rochester, NY, USA) along with <sup>14</sup>C autoradiographic standards for 3 days (PVN, SON) or for 7 days [bed nucleus of the stria terminalis (BNST)]. All brain sections were hybridized at the same time and were exposed to the same film to avoid intrinsic variations between different *in situ* hybridizations and different films. Furthermore, as the density of AVP mRNA expression was much higher than the <sup>14</sup>C standard scale, we used different exposure times in order to verify that the optical density values were within the linear range of the film. Using the NIH Image program (ImageJ 1.31, National Institute of Health, <http://rsb.info.nih.gov/ij/>), the optical density of AVP mRNA expression in the magnocellular part of the PVN (mPVN), the SON and in the BNST was determined bilaterally in the brain slices with the highest density of mRNA expression. This resulted in bilateral measurements in two–four brain sections per region of interest for each rat, which were pooled to provide an average per brain area per rat. For tissue background, the optical density of a non-hybridized region outside the mPVN, SON or BNST was measured.

### Experiment 3: MS effects on aggression and on AVP and 5-HT immunoreactivity

#### Aggression

At the age of 14 weeks, MS ( $n = 11$ ) and control ( $n = 8$ ) male rats were tested for intermale aggression during three 10-min RI tests carried out on consecutive days.

#### AVP and 5-HT immunocytochemistry

To measure the effects of RI exposure on hypothalamic AVP and 5-HT immunoreactivity, MS ( $n = 11$ ) and control ( $n = 8$ ) rats were perfused 2 h after the start of the third RI test. Based on previous studies (Aubry *et al.*, 1999), it was assumed that changes in peptide levels are likely to be more robust after repeated exposure to the RI test. The 2 h time point was chosen based on other studies showing alterations in AVP expression patterns within this time frame following stress exposure (Dent *et al.*, 2000; Givalois *et al.*, 2004; Itoi *et al.*, 2004; Kawasaki *et al.*, 2005; McDougall *et al.*, 2005). Another group of MS ( $n = 10$ ) and control ( $n = 8$ ) rats were perfused under the same conditions without preceding RI tests, in order to measure AVP and 5-HT immunoreactivity under basal conditions. All rats received CO<sub>2</sub> anaesthesia and were perfused transcardially with 150 mL ice-cold phosphate-buffered saline (PBS), followed by 300 mL 4% paraformaldehyde in ice-cold phosphate buffer (pH 7.4). The brains were postfixed overnight, transferred into 30% sucrose and stored at 4  $^{\circ}\text{C}$ . Forty-micrometre cryocut coronal sections were stored at 4  $^{\circ}\text{C}$  in 0.1 M phosphate buffer. For immunocytochemistry, brain sections were matched for level among the groups, and the same number of brain slices for each animal was used.

AVP immunocytochemistry was adopted from Dai *et al.* (1997). Briefly, free-floating sections were washed in TBS (50 mM Tris/150 mM NaCl, pH 7.6), following incubation overnight with rabbit anti-AVP (Truus, 29-01-86, Netherlands Institute for Brain Research, 1 : 2000) in supermix (TBS + 0.25% gelatine + 0.5% Triton, pH 7.6). After rinsing in TBS, sections were incubated with biotinylated goat-anti-rabbit IgG (Vector, 1 : 400) for 1 h, followed by ABC complex (Vector, 1 : 800) and diaminobenzidine (DAB, 0.25 mg/mL, 0.01% H<sub>2</sub>O<sub>2</sub>).

5-HT immunocytochemistry was adopted from Nyakas *et al.* (1994). Briefly, free-floating sections were washed in PBS (0.01 M, pH 7.4), incubated in 0.3% H<sub>2</sub>O<sub>2</sub> for 30 min, preincubated for 1 h in 5% normal horse serum, 0.4% TX-100 dissolved in PBS, following incubation for 3 days with mouse anti-5-HT (1 : 100 000, kindly donated by Dr L. Léger, Lyon, France). After rinsing in PBS, sections were incubated with biotinylated horse-anti-mouse IgG (Vector, 1 : 500) overnight, followed by ABC complex (Vector, 1 : 500) and DAB (0.25 mg/mL DAB, 0.01% H<sub>2</sub>O<sub>2</sub>).

Sections were mounted onto slides, air dried and coverslipped the following day. The optical density of AVP- and 5-HT-immunoreactive staining was quantified as grey density per area minus background in digitized images using IMAGE software and obtained from a video camera mounted on a microscope. The sections were obtained with a 10  $\times$  lens to maximize capture of the areas of interest. The images were imported on a MacIntosh computer equipped with a frame grabber. The following hypothalamic brain areas were analysed for AVP: the mPVN, the posterior part of the PVN (PaPo), the SON, the nucleus circularis and the lateral hypothalamus. The following brain areas were analysed for 5-HT: the anterior hypothalamus, the SON, the lateral hypothalamus, the dorsomedial hypothalamic nucleus and the basolateral amygdala. Background measurements were taken from tissue lateral to the analysed region that exhibited no evident signal. The results are expressed as the optical density of immunoreactive signal within the sample area with a diameter circle of 50  $\mu\text{m}$  (nucleus circularis), 200  $\mu\text{m}$  (PVN, SON) or 250  $\mu\text{m}$  (lateral, anterior and dorsomedial hypothalamus, basolateral amygdala). Bilateral measurements were taken for each rat in those brain slices with the highest density of immunoreactive staining. This resulted in bilateral measurements in two–four brain sections per region of interest for each rat, which were averaged per brain area per rat.

## Statistical analysis

Behaviour in the forced swim test was analysed with a one-way ANOVA. An ANOVA for repeated measures was used for analysing plasma ACTH, corticosterone and testosterone concentrations (factor MS  $\times$  factor time) and behaviour during the RI tests (factor MS  $\times$  factor time). Two-way ANOVA (factor MS  $\times$  factor RI) was used to analyse AVP mRNA expression and AVP and 5-HT immunoreactivity. When appropriate, ANOVA was followed by a Bonferroni *post hoc* test. Correlation analysis of aggressive behaviour (including all scored parameters) with AVP or 5-HT immunoreactivity in the brain regions measured was carried out using simple regression analysis, with a Bonferroni correction for multiple comparisons. For all tests the software package SPSS (version 12) was used. Data are presented as mean  $\pm$  SEM. Significance was accepted at  $P < 0.05$ .

## Results

### Experiment 1: MS effects on behavioural and neuroendocrine reactivity

#### Forced swim behaviour

MS rats displayed less swimming ( $P < 0.05$ ) and more immobility ( $P < 0.01$ ) and had a shorter latency time to show immobility ( $P < 0.05$ ) than control rats, indicating a more pronounced passive stress-coping in MS rats (Table 1).

TABLE 1. Behavioural performance of adult control ( $n = 8$ ) and maternally separated (MS,  $n = 10$ ) male rats during the forced swim test

	Control	MS
Immobility latency (s)	198.9 $\pm$ 36.5	112.7 $\pm$ 21.4*
Immobility (time, %)	3.4 $\pm$ 0.9	14.0 $\pm$ 2.7**
Swimming (time, %)	74.9 $\pm$ 1.7	63.4 $\pm$ 3.3*
Climbing (time, %)	21.7 $\pm$ 2.0	22.6 $\pm$ 1.5

Data are mean  $\pm$  SEM, \* $P < 0.05$  and \*\* $P < 0.005$ , vs. control, ANOVA.

## ACTH

ANOVA indicated main effects for time ( $F_{6,78} = 61.38$ ,  $P < 0.001$ ) and treatment ( $F_{1,13} = 4.85$ ,  $P < 0.05$ ). Exposure to 5 min forced swimming elevated plasma ACTH concentrations compared with basal ACTH concentrations in control and MS rats. MS rats, however, showed significantly higher ACTH concentrations 5 min after the onset of the swim stressor ( $P < 0.05$ ) and a tendency toward higher ACTH concentrations at 15 min ( $P = 0.053$ ) compared with control rats (Fig. 1A). In MS rats, plasma ACTH concentrations remained elevated until 60 min after the onset of the stressor compared with basal ACTH concentrations ( $P < 0.05$ ). In contrast, in control rats, plasma ACTH concentrations declined already to baseline at 30 min. Basal plasma ACTH concentrations did not differ between MS and control rats.

## Corticosterone

ANOVA indicated a main effect only for time ( $F_{6,78} = 27.56$ ,  $P < 0.001$ ). Exposure to forced swimming elevated plasma corticosterone concentrations compared with basal corticosterone concentrations in control and MS rats. In MS rats, plasma corticosterone concentrations were elevated 5 min after the onset of the stressor ( $P < 0.05$ ), whereas in control rats corticosterone concentrations were elevated 15 min after the onset of the stressor ( $P < 0.05$ ; Fig. 1B). Basal as well as stress-induced plasma corticosterone concentrations were not significantly different between MS and control rats.

## Testosterone

ANOVA indicated a main effect for time ( $F_{1,13} = 12.65$ ,  $P < 0.005$ ). Only MS rats showed a decrease in plasma testosterone concentrations upon swim stress-exposure compared with basal testosterone concentrations ( $P < 0.005$ ; Fig. 1C).

### Experiment 2: MS effects on AVP mRNA expression

For both the PVN and SON, main effects were found for MS (PVN:  $F_{1,28} = 6.24$ ,  $P < 0.05$ ; SON:  $F_{1,28} = 4.96$ ,  $P < 0.05$ ) and for

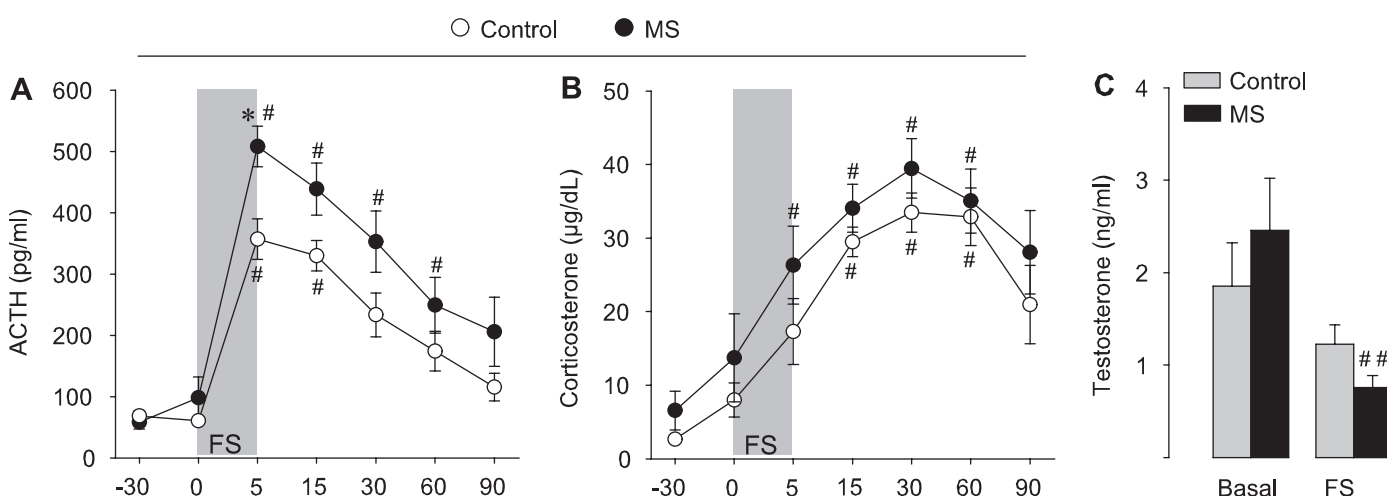


FIG. 1. Effects of maternal separation (MS) on basal and stress-induced plasma adrenocorticotropin hormone (ACTH) (A), corticosterone (B) and testosterone (C) concentrations. For plasma ACTH and corticosterone concentrations, blood samples were taken from freely moving control and MS rats under basal conditions (−30 and 0 min), and at several time points after 5 min of forced swim stress (FS). Plasma testosterone concentrations were measured under basal conditions (0 min) and 30 min after FS. Data are means  $\pm$  SEM. \* $P < 0.01$  vs. control rats, # $P < 0.05$ , ## $P < 0.005$  vs. respective basal samples, ANOVA for repeated measures followed by Bonferroni *post hoc* test.

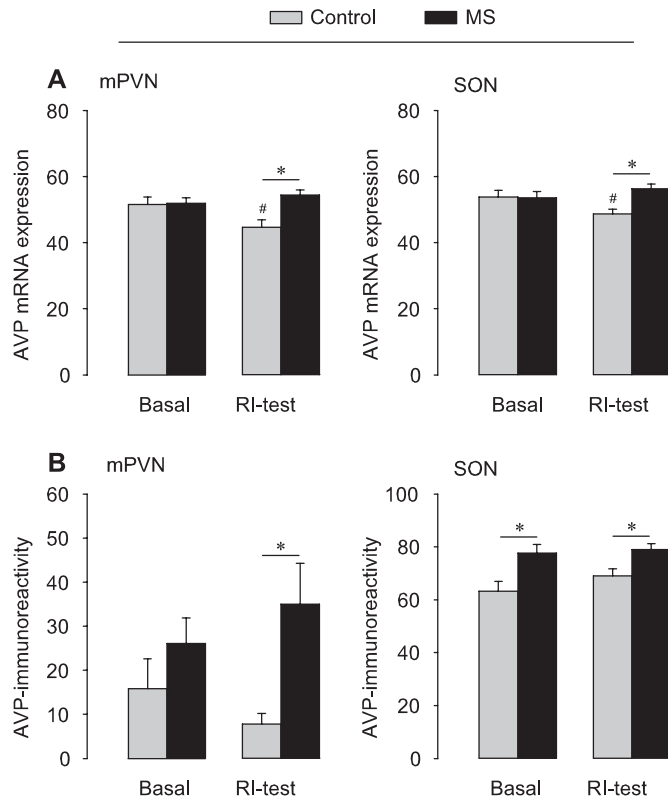


FIG. 2. Effects of maternal separation (MS) on the optical density (arbitrary units) of (A) arginine vasopressin (AVP) mRNA expression in the magnocellular part of the paraventricular nucleus of the hypothalamus (mPVN) and supraoptic nucleus (SON) under basal conditions and 1 h after the resident-intruder (RI) test, and (B) AVP-immunoreactive cell bodies in the mPVN and the SON under basal conditions and 2 h after the RI test. Data are means + SEM. \* $P < 0.05$ , # $P \leq 0.05$  vs. representative basal group, ANOVA, followed by Bonferroni *post hoc* test.

MS  $\times$  RI exposure (PVN:  $F_{1,28} = 5.42$ ,  $P < 0.05$ ; SON:  $F_{1,28} = 5.61$ ,  $P < 0.05$ ). Basal AVP mRNA expression was not affected by MS in either the PVN or SON (Figs 2A and 6). Control rats showed a decrease in AVP mRNA expression in both the mPVN and SON upon RI exposure ( $P < 0.05$ ), whereas MS rats did not show this RI-induced decline. As a result, a higher AVP mRNA expression was found in both hypothalamic nuclei in MS compared with control rats 1 h after RI exposure ( $P < 0.005$ ). No effects of MS or RI test exposure on AVP mRNA expression in the BNST were found (optical density values: control basal,  $21.2 \pm 2.6$ ; MS basal,  $23.3 \pm 2.1$ ; control RI,  $22.1 \pm 3.1$ ; MS RI,  $26.4 \pm 2.8$ ).

### Experiment 3: MS effects on aggression and on AVP and 5-HT immunoreactivity

#### Aggression

Using ANOVA for repeated measures, no time effect was found for any of the behavioural parameters recorded. Therefore, the mean was calculated for each behavioural parameter per rat over the three RI tests. One-way ANOVA revealed that MS resident males displayed more aggressive behaviour than control resident males ( $P < 0.05$ , Fig. 3A). Among the behavioural elements of aggression, MS rats showed significantly more lateral threat ( $P < 0.05$ ), offensive upright ( $P < 0.01$ ) and keep down ( $P < 0.05$ , Fig. 3B). No differences were found for the attack latency time (Fig. 3A) or

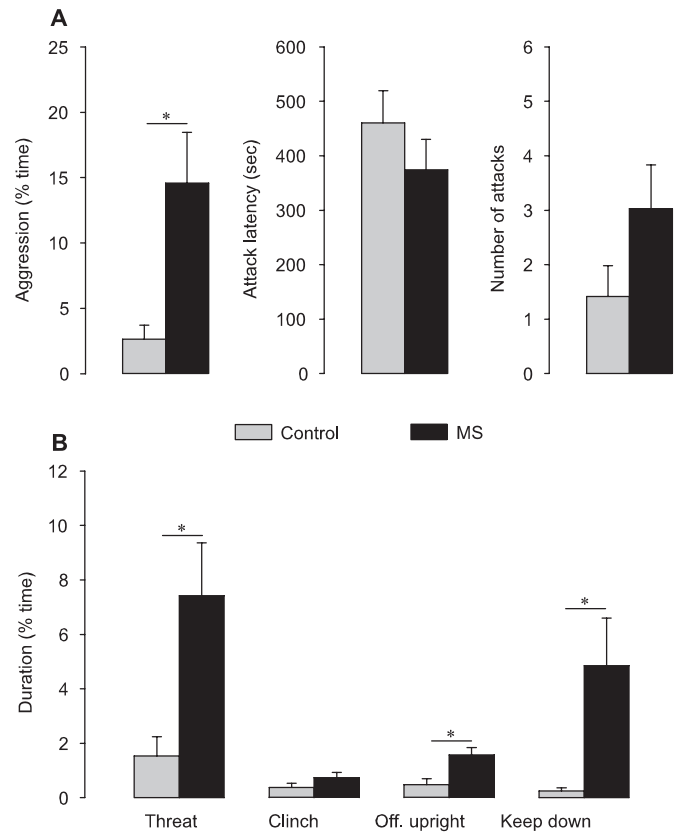


FIG. 3. Effect of maternal separation (MS) on (A) the percentage time of total aggressive behaviour, the attack latency time, the number of attacks, and (B) the percentage time of the detailed behavioural elements of aggressive behaviour during exposure to the RI test. Data are means + SEM. \* $P < 0.05$ , ANOVA followed by Bonferroni *post hoc* test.

for the number of attacks (Fig. 3A). Also, no differences were found for the percentage time of social behaviour (control:  $40.5 \pm 6.0\%$ ; MS:  $38.9 \pm 3.5\%$ ), exploration (control:  $43.1 \pm 4.3\%$ ; MS:  $35.9 \pm 2.7\%$ ) or self grooming (control:  $8.3 \pm 1.5\%$ ; MS:  $7.2 \pm 2.0\%$ ).

#### AVP immunoreactivity

Exposure to MS significantly increased the optical density of AVP-immunoreactive staining in adult male rats in the PVN (mPVN and PaPo, only after RI test exposure), the SON and the lateral hypothalamus (Figs 2B and 6; for statistical details, see Table 2). RI exposure was not accompanied by a significant change in AVP immunoreactivity in any of the brain regions tested. Despite that, a tendency toward a general decrease in AVP immunoreactivity in the hypothalamic regions measured was seen in control rats, while MS rats rather showed an increase (Table 2). No correlation was found between any of the parameters of intermale aggression and the optical density of AVP-immunoreactive staining in any of the hypothalamic regions.

#### 5-HT immunoreactivity

MS reduced the optical density of 5-HT-immunoreactive staining only in the anterior hypothalamus of adult male rats, both under basal conditions and after RI exposure (for statistical details, see Table 3). No effect of MS or RI exposure was found for 5-HT immunoreactivity

TABLE 2. AVP-immunoreactive staining in hypothalamic areas of male adult controls and maternally separated male rats: basal and after the RI test

Hypothalamic areas	Optical density of AVP-immunoreactivity (arbitrary units)					
	Basal		2 h after exposure to RI test		MS effects	
	Control ( <i>n</i> = 8)	MS ( <i>n</i> = 8)	Control ( <i>n</i> = 9)	MS ( <i>n</i> = 11)	<i>F</i> <sub>1,32</sub>	<i>P</i> -value
PaPo	12.3 ± 2.5	17.1 ± 2.9	8.0 ± 2.2	20.5 ± 3.1*	9.14	0.005
NC	13.0 ± 2.4	20.1 ± 6.9	10.4 ± 4.2	25.4 ± 6.4	3.67	0.064
LH	15.2 ± 1.3	19.7 ± 1.3*	13.7 ± 0.8	20.0 ± 1.4*	17.31	0.001

Data are mean ± SEM. LH, lateral hypothalamic area; NC, nucleus circularis; PaPo, posterior part of the PVN. *F*<sub>1,32</sub>, ANOVA for MS effects, \**P* < 0.05 vs. respective control, Bonferroni *post hoc* test.

TABLE 3. Optical density of 5-HT-immunoreactive fibres and varicosities in several brain regions of adult control and maternally separated (MS) male rats under basal conditions or 2 h after RI test exposure

	Optical density of 5-HT-immunoreactivity (arbitrary units)					
	Basal		RI test		MS effects	
	Control	MS	Control	MS	<i>F</i> <sub>1,32</sub>	<i>P</i> -value
AH	44.2 ± 4.9	26.4 ± 6.9*	41.7 ± 3.3	15.5 ± 3.9*	21.7	0.001
SON	35.0 ± 4.1	32.7 ± 3.8	39.4 ± 2.6	34.2 ± 3.5	1.18	0.287
LH	32.6 ± 4.8	34.8 ± 4.0	32.2 ± 3.2	39.9 ± 4.3	1.35	0.254
DMH	45.7 ± 4.8	32.3 ± 4.5	36.6 ± 6.0	42.0 ± 4.6	0.62	0.439
BLA	91.0 ± 7.1	72.8 ± 7.7	75.3 ± 7.5	89.3 ± 8.1	0.07	0.080

Data are expressed as mean ± SEM. AH, anterior hypothalamus; BLA, basolateral amygdala; DMH, dorsomedial hypothalamic nucleus; LH, lateral hypothalamic area; SON, supraoptic nucleus. *F*<sub>1,32</sub>, ANOVA for MS effects, \**P* < 0.05 vs. respective control, Bonferroni *post hoc* test.

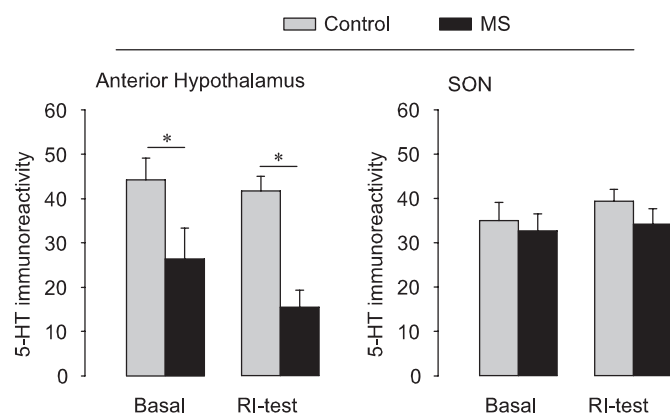


FIG. 4. Effect of maternal separation (MS) on the optical density (arbitrary units) of serotonin (5-HT)-immunoreactive fibres in the anterior hypothalamus and the supraoptic nucleus (SON) under basal conditions and 2 h after exposure to the resident-intruder (RI) test. \**P* < 0.05.

in any of the other brain regions analysed (Figs 4–6; Table 3). A negative correlation was found between the duration of lateral threat and the optical density of 5-HT fibres in the anterior hypothalamus ( $r^2 = 0.562$ ,  $P = 0.035$ ) and the SON ( $r^2 = 0.502$ ,  $P = 0.035$ ) (Fig. 5). No correlations were found between any of the other parameters of intermale aggression and 5-HT immunoreactivity in any of the brain regions measured.

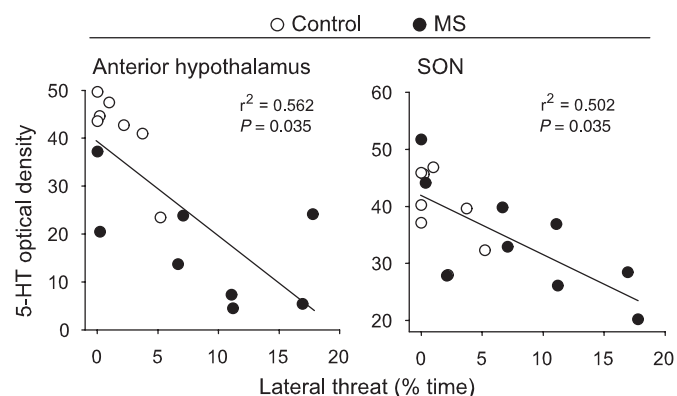


FIG. 5. Correlation of the duration of lateral threat displayed by control and maternally separated (MS) adult male rats during the RI tests with the optical density of serotonin (5-HT)-immunoreactive fibres in the anterior hypothalamus and in the supraoptic nucleus (SON).

## Discussion

Despite the fact that in existing human societies a link between early life trauma and disturbances in adult aggression seems to be generally accepted, animal models studying this phenomenon have been lacking until now. In the present study, we show that exposure to early life stress significantly increased adult aggression of male Wistar rats. The MS-induced increase in male aggression was accompanied by a significant higher AVP mRNA expression and AVP-immunoreactive staining in the mPVN and SON upon RI test exposure compared with control rats. In contrast, a significant decrease in 5-HT immunoreactivity in the anterior hypothalamus was found after MS. Importantly, there was a significant negative correlation between 5-HT-immunoreactive staining in the anterior hypothalamus as well as in the SON and the duration of lateral threat.

We confirmed MS-induced long-term changes in behavioural stress-coping and HPA axis responsiveness. Adult MS rats displayed a rather passive stress-coping style, indicated by enhanced immobility, and enhanced and prolonged plasma ACTH secretion in response to forced swimming. These findings are in agreement with other rodent studies demonstrating that repeated separation from the dam during the first 2 weeks of life resulted in increased passive behaviours, increased anxiety- and depression-like behaviours, and in elevated ACTH responses to an acute stressor (Plotsky & Meaney, 1993; Wigger & Neumann, 1999; Caldji *et al.*, 2000; Ladd *et al.*, 2000; Huot *et al.*, 2002; Kalinichev *et al.*, 2002; Gardner *et al.*, 2005).

Despite the fact that MS rats displayed a rather passive coping style during forced swimming, which is indicative of depression-like



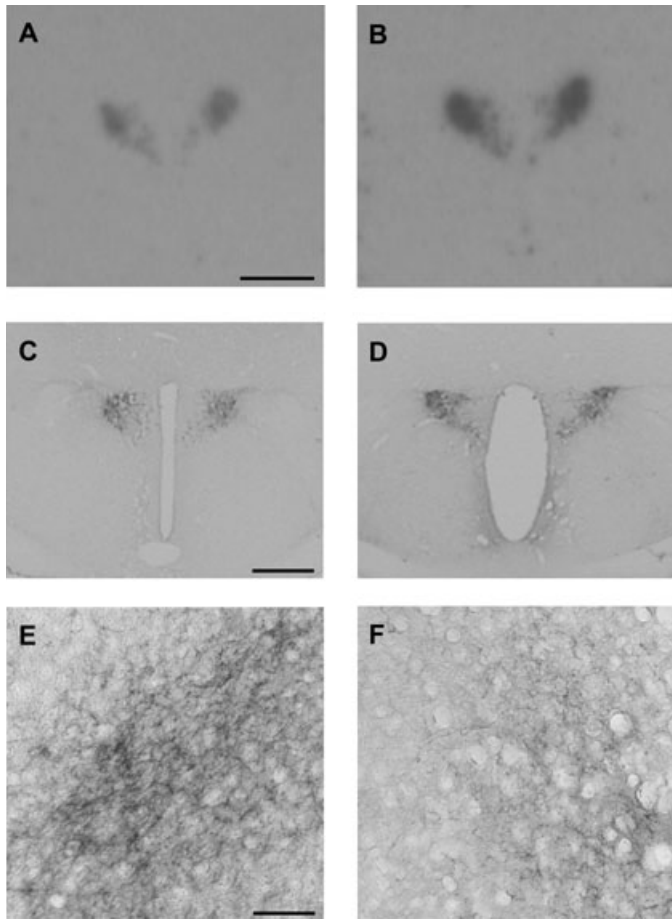


FIG. 6. Representative images of coronal sections of the hypothalamic region representing AVP mRNA expression (A and B) and AVP-immunoreactive staining (C and D) in the magnocellular part of the paraventricular nucleus, and 5-HT-immunoreactive staining in the anterior hypothalamus (E and F) comparing control (A, C and E) and maternally separated (B, D, F) adult male rats exposed to the RI test. Scale bar, 500  $\mu$ m (A and C) or 200  $\mu$ m (E).

behaviour (Porsolt, 1979; Cryan & Mombereau, 2004), they show significantly more aggressive behaviour when confronted with a male intruder in their home-cage than control rats. The elevated level of aggression in MS rats was specifically reflected by the display of more lateral threat, offensive upright and keep down. Interestingly, clinical studies also have shown that psychopathologies such as depressed mood, anxiety, impulsivity and aggression dysregulation are closely related and may even be correlated (Van Praag, 1986; Apter *et al.*, 1990; Van Praag, 1998, 2001).

Underlying mechanisms accounting for the MS-induced increase in adult male aggression may include changes in HPA responsiveness. Activation of the HPA axis modulates aggressive behaviour (Haller *et al.*, 1998, 2001). An acute rise in plasma corticosterone concentrations promotes aggression (Mikics *et al.*, 2004), and may contribute to the escalation of violent behaviour under stressful conditions (Kruk *et al.*, 2004). ACTH was also found to stimulate aggressive behaviour, via glucocorticoid effects on the brain or via direct effects on the brain (Brain & Evans, 1977; Clarke & File, 1983). Although HPA axis responsiveness to an aggressive encounter was not quantified, the elevated ACTH response to forced swimming in MS rats indicates changes in the HPA axis regulation, which may have contributed to their higher level of aggression.

It should also be considered that the more pronounced ACTH responsiveness in MS rats may indicate MS-induced changes in

relevant neuronal systems, which, in turn, might have played a role in the behavioural changes observed in MS rats. Indeed, exposure to postnatal stress was shown to increase CRH gene transcription in the PVN (Plotsky & Meaney, 1993; Ladd *et al.*, 1996), to decrease glucocorticoid receptor mRNA levels in the hippocampus and medial prefrontal cortex, and to increase mineralocorticoid receptor mRNA levels in the hippocampus (Meaney *et al.*, 1996; Ladd *et al.*, 2004). MS also increased basal and stress-induced norepinephrine release in the PVN (Liu *et al.*, 2000), and induced changes in  $\gamma$ -aminobutyric acid (GABA)/benzodiazepine receptor binding in several brain regions (Caldji *et al.*, 2000). In addition, the present study showed long-lasting alterations in the regulation of AVP synthesis in different hypothalamic regions after MS. Although basal AVP mRNA expression within the mPVN and SON did not differ between groups, exposure to a single RI test was associated with a significant decrease in AVP mRNA expression in control, but not in MS, rats. Consequently, MS rats showed a significantly higher AVP mRNA expression in these regions after a single RI test exposure compared with control rats, which was also reflected by a higher AVP immunoreactivity after repeated exposure to the RI test.

Previous studies have indicated an important role for AVP within the brain regulating neuroendocrine and behavioural stress-coping (Bielsky *et al.*, 2004; Engelmann *et al.*, 2004; Landgraf & Neumann, 2004; Griebel *et al.*, 2005). At the level of the PVN and SON, locally released AVP regulates ACTH release from the pituitary (Antoni, 1993; Wotjak *et al.*, 1996, 2002). Infusion of an AVP V1-receptor antagonist into the PVN of rats bred for high trait anxiety resulted in a decrease in anxiety and in a switch from passive to active behavioural coping in the forced swim test (Wigger *et al.*, 2004). Furthermore, after prenatal stress, an altered expression profile of AVP has also been found within the hypothalamus (Bosch *et al.*, 2006). In addition, several human and animal studies suggest a positive relationship between brain AVP and aggression (Ferris, 1992; Ferris *et al.*, 1997; Coccaro *et al.*, 1998; Stribley & Carter, 1999). In patients with personality disorders, cerebral spinal fluid concentrations of AVP were found to be positively correlated with a life history of aggressive behaviour (Coccaro *et al.*, 1998). In aggressive male hamsters, AVP neurons in the SON showed increased neuronal activity (Delville *et al.*, 2000), and have been identified as innervating the anterior hypothalamus, which is also involved in offensive aggression (Ferris, 1992; Ferris *et al.*, 1997). In addition, the decreased aggression in highly aggressive California mice after cross-fostering with less aggressive white-footed mice was associated with decreased SON AVP immunoreactivity (Bester-Meredith & Marler, 2001). MS also elevated the density of AVP-immunoreactive staining in the lateral hypothalamus. This brain region contains AVP V1A-receptor binding sites (Tribollet *et al.*, 1998), and has been reported to be involved in the modulation of aggression (Koolhaas, 1978; Kruk *et al.*, 1983; Gregg & Siegel, 2001; Gregg, 2003). In the rat, electrical stimulation of the lateral hypothalamus induces intraspecific aggressive behaviour in the presence of a subordinate rat (Koolhaas, 1978). Taken together, the MS-induced alterations in AVP synthesis in the mPVN, SON and lateral hypothalamus may have contributed, among other factors, to changes in behavioural stress-coping, including aggressive behaviour. It would be of further interest to study whether the general responsiveness of the hypothalamic AVP system to stressful stimuli is altered after MS, or whether the altered responsiveness is selectively restricted to exposure to an aggressive encounter.

Besides MS-induced changes in AVP in hypothalamic nuclei, it is likely that MS affected AVP in extrahypothalamic nuclei as well. In particular, the AVP pathway originating in the BNST and medial amygdala, and projecting to the lateral septum is of interest, as this



pathway has been associated with aggression (Koolhaas *et al.*, 1998; Bester-Meredith *et al.*, 1999; Bester-Meredith & Marler, 2001; D'Eath *et al.*, 2005). In contrast to the mPVN and SON, exposure to MS or exposure to the RI test did not modulate AVP mRNA expression in the BNST. This may indicate site-specific effects of MS on central AVP mRNA expression. Yet, other factors, like testosterone, might regulate the central AVP system at the peptide level (Wang & De Vries, 1993; De Vries *et al.*, 1994; Delville *et al.*, 1996b). Alterations in plasma testosterone were found in MS rats after forced swimming. Future studies will need to address whether MS modulated plasma testosterone responses to RI test exposure, and whether this affected the activity of the AVP system.

In contrast to the AVP system, the density of 5-HT-immunoreactive fibres in the anterior hypothalamus was found to be significantly reduced after MS. Moreover, 5-HT immunoreactivity in the anterior hypothalamus and in the SON correlated negatively with the duration of lateral threat, which is an important element of aggressive behaviour. The latter finding agrees with the general view that 5-HT exerts an inhibitory control over impulsive aggression (Olivier & Mos, 1990; Ferris & Delville, 1994; Ferris, 1996; Ferris *et al.*, 1997). In humans, excessive aggression and impulsive violent behaviours are associated with diminished central 5-HT function, as shown by low cerebrospinal fluid levels of the 5-HT metabolite 5-hydroxyindolacetic acid (Brown & Linnoila, 1990; Kruesi *et al.*, 1990). Our finding is also in accordance with studies in hamsters showing that 5-HT exerts an inhibitory effect on intermale aggression at the level of the anterior hypothalamus and the SON (Ferris, 1996; Ferris *et al.*, 1997). This effect is likely mediated through postsynaptic 5-HT<sub>1A</sub> receptors (Ferris *et al.*, 1999), and involves inhibition of the AVP system in the anterior hypothalamus (Ferris *et al.*, 1997). The MS-induced decrease in 5-HT-immunoreactive fibres in the anterior hypothalamus suggests a decrease in 5-HT release in this specific brain region. Interestingly, in hamsters that show abnormal aggressive behaviour (increased aggression towards smaller intruders, decreased aggression towards equal size intruders) after being 'abused' during puberty, the number of 5-HT terminals within the anterior hypothalamus was found to be increased (Delville *et al.*, 1998). Taken together, a balanced activity of the 5-HT system within the anterior hypothalamus seems to be critical for the regulation of appropriate aggressive behaviour.

It should be noted that it is difficult to conclude whether the changes in hypothalamic AVP mRNA expression and in hypothalamic AVP and 5-HT immunoreactivity observed after MS are associated with an actual change in local AVP and 5-HT release, respectively. Future studies will have to examine whether the increase in aggressive behaviour observed after MS is associated with altered hypothalamic AVP and/or 5-HT release with more delicate techniques such as microdialysis.

In summary, our data provide the first evidence that exposure to early life stress increased adult male aggression, which was associated with changes in the activity of hypothalamic AVP and 5-HT systems. Studying the mechanisms underlying MS-induced changes in aggressive behaviour may contribute to understanding the complex interaction between early rearing conditions and the neurobiological factors regulating aggression. As aggression causes major public health and social problems, these studies seem to be essential for the future prevention and management of excessive aggression.

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## Abbreviations

5-HT, 5-hydroxytryptamine (serotonin); ACTH, adrenocorticotropin hormone; AVP, arginine vasopressin; BNST, bed nucleus of the stria terminalis; CRH, corticotropin-releasing hormone; DAB, diaminobenzidine; HPA, hypothalamic-pituitary-adrenocortical; mPVN, magnocellular part of the PVN; MS, maternal separation; PaPo, posterior part of the PVN; PBS, phosphate-buffered saline; PVN, paraventricular nucleus of the hypothalamus; RI, resident-intruder; SON, supraoptic nucleus; SSC, standard sodium citrate.

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